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Note

Evaluating the intrinsic capacity of oral bacteria to produce hydrogen peroxide (H_2O_2) in liquid cultures: Interference by bacterial growth media

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ABSTRACT

This work highlights the issue of interference by growth media when measuring bacterial H_2O_2 production. H_2O_2 was shown to be stable in phosphate buffered saline (PBS) but not in growth media. The protocol used for evaluating the intrinsic capacity of oral streptococci to produce H_2O_2 was shown to be reliable.

Hydrogen peroxide (H_2O_2) production is an inherent feature of the commensal oral microbiota, more specifically of certain streptococci that possess highly conserved pathways through which they produce large amounts of H_2O_2 under aerobic conditions (Redanz et al., 2018a, 2018b; Garcia-Mendoza et al., 1993; Zhu and Kreth, 2012; Herrero et al., 2016). On the contrary, for instance periodontal pathogens lack the ability to produce H_2O_2 and generally show high sensitivity to H_2O_2-mediated toxicity (Zhu and Kreth, 2012; Redanz et al., 2018a; Herrero et al., 2016). H_2O_2 can exert a broad range of effects in the environment, where it can for instance inhibit neighbouring competing species and regulates biofilm development (Redanz et al., 2018a, 2018b; Erttmann and Gekara, 2019; Thurnheer and Belibasakis, 2018). Therefore, the role of H_2O_2 in the oral cavity has been intensively studied and characterized. However, given the highly reactive and unstable nature of H_2O_2 under biological conditions (Pryor, 1986; Winterbourn, 2013), measurement of H_2O_2 levels can be challenging. Especially when conducting fundamental, proof-of-concept research, it is important to measure actual H_2O_2 levels in an accurate way. Such measurements are often done directly/indirectly using bacterial colonies grown on agar plates, but less frequently in liquid cultures. This work aims to increase the awareness that the type of growth medium can interfere with the determination of H_2O_2 levels in liquid cultures. Furthermore, it evaluates the applicability of a slightly modified version of a previously described protocol designed for *Streptococcus pneumoniae* to reliably measure H_2O_2 production by oral streptococci (Pesakhov et al., 2007).

To evaluate the effect of liquids in which experiments are performed, a concentration range (0 μM to 10 μM) was prepared in phosphate buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO4, 0.24 g/L KH_2PO_4, pH 7.4), PBS + 0.5 mM glucose, Brain Heart Infusion broth (BHI) (Difco Laboratories, Detroit, USA), Tryptic Soy broth (TSB) or Tryptic Soy broth without Dextrose (TSB^-) (both Becton Dickinson, Franklin Lakes, USA), starting from a stabilized 30%$_{(w/w)}$ H_2O_2 stock solution (Sigma-Aldrich Co, St. Louis, USA). All media were freshly prepared from the same respective batches. H_2O_2 concentrations were determined using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, USA), with a linear range of 0 μM to 10 μM.

To determine the optimal experimental conditions for measuring oral streptococcal H_2O_2 production, a slightly modified version of a previously described protocol, where *S. pneumoniae* H_2O_2 production was measured in PBS to minimize the Fenton reaction, was used (Pesakhov et al., 2007; Imlay, 2003). In a first experiment, the 30%$_{(w/w)}$ H_2O_2 stock was diluted to 100 μM in PBS, PBS + 0.5 mM glucose, BHI, TSB or TSB^- (TSB without glucose). In a second experiment, overnight aerobic cultures (BHI, 37 °C, 5% CO_2) of *Streptococcus oralis* DSM 20627 and *Streptococcus mitis* DSM 12643 were adjusted to OD_{600} 0.05 in fresh BHI in an Erlenmeyer and grown aerobically (37 °C, 5% CO_2, 220 rpm) until mid-log phase. Aliquots were taken, centrifuged (5 min, 7179 ×*g*),

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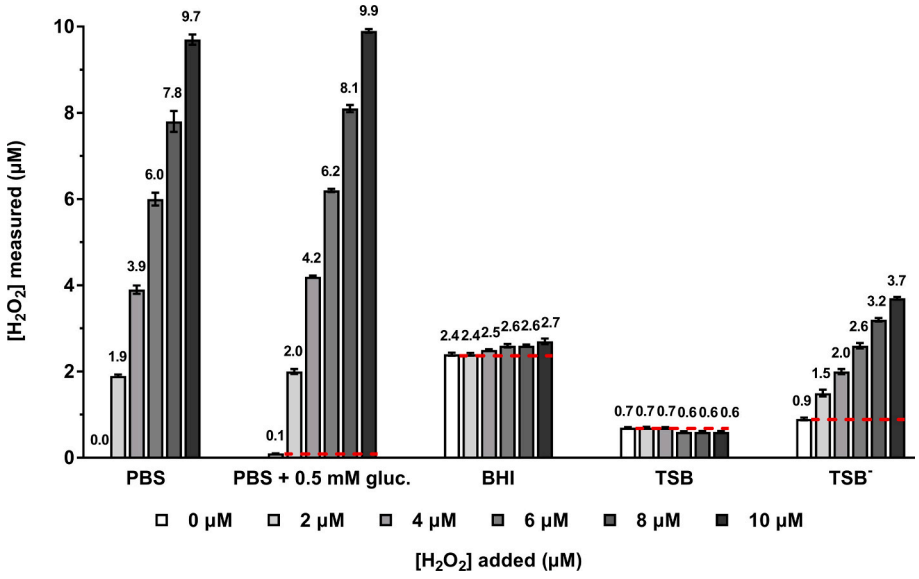

Fig. 1. Calibration curves of hydrogen peroxide (H_2O_2) in PBS and different media.
A concentration range of H_2O_2, ranging from 0 to 10 μM, was prepared in either PBS, PBS + 0.5 mM glucose, BHI, TSB or TSB$^-$. H_2O_2 concentrations ([H_2O_2]) were subsequently determined and are shown as mean ± SD values of three independent replicates (n = 3). All values were corrected for the background signal of the assay's buffer solution. The red dashed lines indicate the background signals of the different liquids and which [H_2O_2] would remain (i.e. the part above the dashed line) after deducting these background values from the measured values. PBS: phosphate buffered saline; PBS + 0.5 mM gluc.: phosphate buffered saline with 0.5 mM glucose; BHI: brain heart infusion broth; TSB: tryptic soy broth, TSB$^-$: tryptic soy broth without glucose.

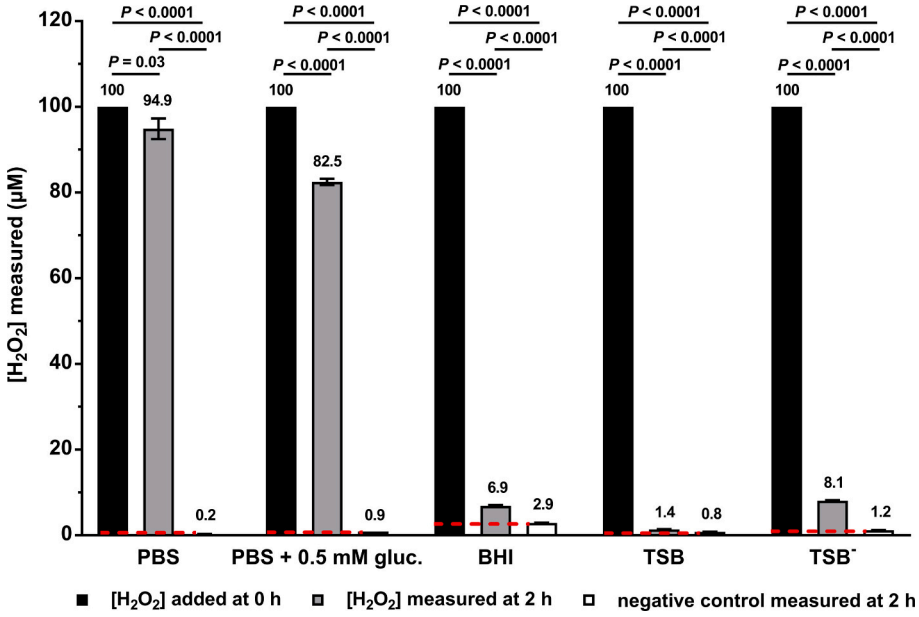

Fig. 2. Stability of hydrogen peroxide (H_2O_2) when performing procedures suitable for experiments involving bacteria.
A concentration of 100 μM H_2O_2 was prepared in either PBS, PBS + 0.5 mM glucose, BHI, TSB or TSB$^-$, followed by a 2 h-incubation, sampling, centrifugation, filter sterilisation and snap freezing. As a negative control, no H_2O_2 was added to the different liquids. H_2O_2 concentrations ([H_2O_2]) were subsequently determined and are shown as mean ± SD values of three independent replicates (n = 3). P-values of statistically significant differences are shown (P < 0.05, ANOVA + Tukey's correction for simultaneous hypothesis testing). All values were corrected for the background signal of the assay's buffer solution. The red dashed lines indicate the background signals of the different liquids (i.e. values for the negative controls) and which [H_2O_2] would remain (i.e. the part above the dashed line) after deducting these background values from the measured values. PBS: phosphate buffered saline; PBS + 0.5 mM gluc.: phosphate buffered saline with 0.5 mM glucose; BHI: brain heart infusion broth; TSB: tryptic soy broth, TSB$^-$: tryptic soy broth without glucose.

and pellets washed with PBS. Following another centrifugation, pellets were resuspended in PBS, PBS + 0.5 mM glucose, BHI, TSB or TSB$^-$. For both experiments, an aerobic incubation of 2 h (37 °C, 220 rpm, 5% CO_2) was followed by sampling and centrifugation (10 min, 4 °C, 10000 ×g), after which the supernatant was filter sterilised and snap frozen in liquid nitrogen. Samples were stored (−80 °C) and later analysed as described earlier. Since bacterial growth was anticipated to occur in BHI/TSB/TSB$^-$ but not in PBS/PBS + glucose, OD_{600} values of the samples were determined before (0 h) and after (2 h) incubation. To allow for comparison, H_2O_2 levels were normalized for OD_{600} values.

The results obtained for the H_2O_2 concentration range in different liquids indicate that H_2O_2 is stable in a non-interfering liquid like PBS, but not in growth media (Fig. 1). As can be seen in the blank conditions (0 μM), all three media gave to some extent a background signal, whereas this was not the case for PBS/PBS + glucose. When one would deduct these values from the values of the samples with added H_2O_2, little to no H_2O_2 is detected anymore. Only in TSB$^-$ a stepwise increase in H_2O_2 concentration resulted in an incremental signal, but measured concentrations were much lower than the actual concentrations. Regarding BHI, a previous study reported that 500 μM of H_2O_2 was stable in 50% BHI for 200 min (Ashby et al., 2009). However, this could likely be explained by the higher H_2O_2 concentration and the 1:2 diluted BHI used in that study. Although the exact constitution of the growth media is unknown, it can be expected that H_2O_2 is converted to ROS (reactive oxygen species) in the Fenton reaction and by consequence is very unstable in the growth media. Glucose can be directly oxidized by H_2O_2 and ROS can interact with biomolecules (Mao, 2017; Imlay, 2003; Mello Filho et al., 1984). Other components in the growth media (e.g. proteins and keto acids) might also interact with H_2O_2 (Winterbourn, 2013). When glucose was absent or present at very low concentrations (TSB$^-$ and PBS + glucose, respectively), the instability of H_2O_2 was less pronounced. Therefore, it seems that mainly the absence of glucose in TSB$^-$ versus TSB/BHI might explain the observed differences.

When 100 μM of H_2O_2 in the different liquids underwent the same procedures as when performing an experiment with bacteria, similar results were obtained (Fig. 2). The [H_2O_2] measured in PBS was close to

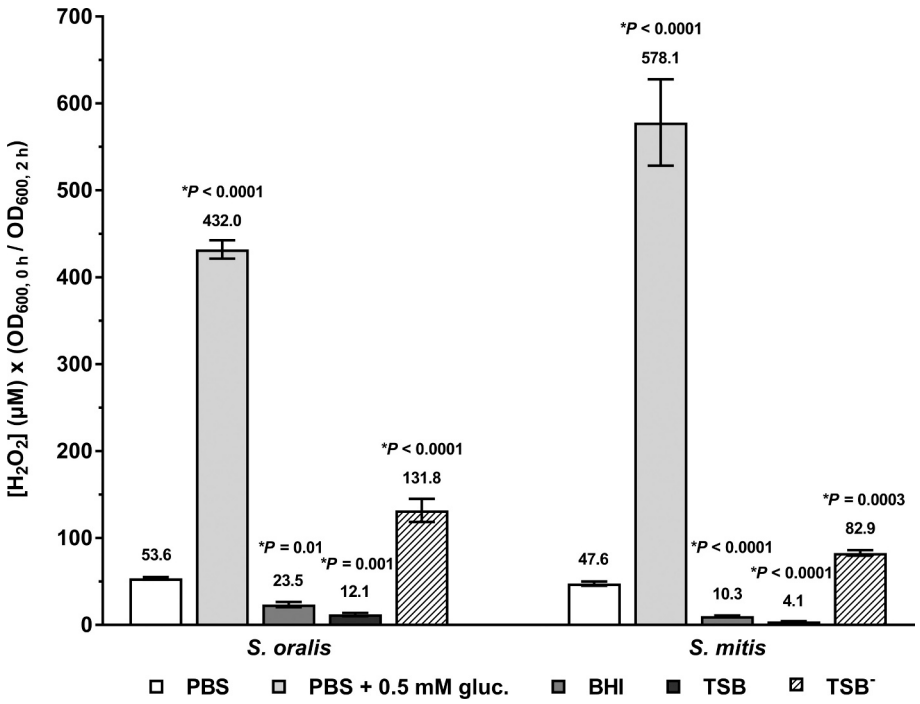

Fig. 3. Oral streptococcal hydrogen peroxide (H_2O_2) production after incubation in different liquids. Pellets of oral streptococcal cultures grown until mid-log phase were washed and resuspended in either PBS, PBS + 0.5 mM glucose, BHI, TSB or TSB$^-$, followed by a 2 h-incubation, sampling, centrifugation, filter sterilisation and snap freezing. H_2O_2 concentrations ($[H_2O_2]$) were subsequently determined, normalized for OD_{600} values, and are shown as mean ± SD values of three independent replicates ($n = 3$). *P*-values of statistically significant differences with the PBS condition are shown ($P < 0.05$, ANOVA + Dunnett's correction for simultaneous hypothesis testing). All values were corrected for the background signal of the assay's buffer solution and for the background signals of the different liquids. *S. oralis*: *Streptococcus oralis*; *S. mitis*: *Streptococcus mitis*; PBS: phosphate buffered saline; PBS + gluc.: phosphate buffered saline with 0.5 mM glucose; BHI: brain heart infusion broth; TSB: tryptic soy broth, TSB$^-$: tryptic soy broth without glucose.

the theoretically added 100 μM, whereas this was not the case for the media. For PBS + glucose, $[H_2O_2]$ was diminished but still relatively close to 100 μM. This indicates that, when performing the 2 h-incubation step in PBS/PBS + 0.5 mM glucose, this experimental set-up is suitable for accurate detection of H_2O_2 levels that are actually present.

Finally, streptococcal H_2O_2 production was evaluated in a similar way (Fig. 3, Supplementary Table A.1). In all conditions, this set-up allowed for the detection of H_2O_2 produced by oral H_2O_2 producers such as *S. oralis* and *S. mitis*. Remarkable was the high $[H_2O_2]$ produced in PBS + 0.5 mM glucose. Since in pure PBS the bacteria will have a maintenance/basal metabolism, the PBS + glucose was included to simultaneously ensure an active metabolism and minimal interference with H_2O_2 (see Figs. 1 and 2). However, when evaluating their capacity to produce H_2O_2 in BHI or TSB, this is most likely an underestimation because of the interferences shown in Figs. 1 and 2. Surprisingly, the TSB$^-$ condition yielded higher H_2O_2 levels (also in comparison with PBS) although a negative effect of the medium can be expected. In BHI and TSB, the bacteria will use glucose as their preferential nutritional source for their growth and metabolic activity (Redanz et al., 2018a, 2018b). H_2O_2 production by some oral species/strains is known to be diminished in presence of high [glucose] due to carbon catabolite repression (CCR) (Redanz et al., 2018a, 2018b). However, the [glucose] in BHI (0.2%) and TSB (0.25%) are likely too low to observe CCR. In addition, since not all of the glucose and other components will be depleted after 2 h, they might still interfere with H_2O_2 that is produced, resulting in lower H_2O_2 levels compared to the PBS condition. However, in TSB$^-$ the bacteria are forced to utilize other components than glucose (e.g. amino acids). This might result in other metabolic pathways that are switched on and that could result in increased H_2O_2 levels that surpass the interference of medium components. After incubation, the pH in TSB$^-$ and PBS was around 7–7.5 and in BHI and TSB around 6.5. Metabolic activity was thus clearly different, but it is unlikely that pH affected the measurement (due to addition of a buffer during the analysis).

Altogether, these data indicate that one should be careful when interpreting data obtained from H_2O_2 measurements in liquid growth media. Such measurements are likely to be underestimations of actually produced H_2O_2 concentrations. This might hamper proper interpretation of results obtained when for instance evaluating intrinsic H_2O_2-producing capacities or when determining bacterial resistance to H_2O_2, as has been highlighted previously (Menschner et al., 2018). When performing proof-of-concept research to evaluate the intrinsic capacity of oral bacteria to produce H_2O_2 in liquid cultures or when trying to boost this H_2O_2 production, it is important to determine H_2O_2 levels as accurately as possible. This work shows that the applied protocol is suitable for doing so, but that it is important to determine H_2O_2 levels in a non-interfering liquid (i.e. with no background interference from ubiquitously present organic compounds) such as PBS.

Conflicts of interest

All authors report no conflicts of interest related to this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106170.

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